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Morphological, Chemical and Antibacterial Characteristics of *Laurus nobilis* L. Growing in Tunisia

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This study shows the variations between ten *Laurus nobilis* L. trees from different Tunisian localities in relation to the morphological parameters, composition of essential oils extracted from leaves and their antibacterial activity. Aggregation of trees according to their geographical origin seems not evident. Two main groups of populations were clearly separated according principally, to the leaves size and the inflorescences number. The leaves essential oil composition did not vary throughout the *Laurus nobilis* L. trees and all analyzed samples were dominated by oxygenated monoterpenes 1,8-cineole, methyl eugenol and α-terpinyl acetate. Specially, oils rich in 1,8-cineole were demonstrated to have potent activities against the *S. aureus* and *E. faecalis*. Individual plant evaluation, however, remains the only way to effectively seek for well defined essential oil composition or specific combinations of morphological and analytical characters.

Keywords: Morphological, Chemical, Antibacterial, *Laurus nobilis* L., Tunisia.

INTRODUCTION

Laurus nobilis L. is a diploid member of the *Lauraceae* family with 2n = 42[1]. It can be found cultivated or naturalized in Mediterranean circumference. In Tunisia, natural laurel (rand) was developed in the edges of rivers and sources of water in mountain and on wet rocks of Ain Draham, Tabarka, Kef and Cap-Bon regions[2]. It is a dioecious plant, with scented male and female flowers. The pollination is entomophilous[3]. The quantitative and qualitative composition of essential oils extracted from stems, leaves, buds and flowers of *L. nobilis* L. varies both between individual plants and between plant parts in this and other species[4]. The highest amounts of essential oil were established in leaves, the lowest, in stems. Commonly predominant essential oil compound in all parts of *L. nobilis* is monoterpene 1,8-cineole, the contents of which can reach up to 33 %[5-7]. The 1,8-cinole is considered as biologically active compound, determining the main pharmacological properties of *L. nobils.* It has been reported in literature that fruits of laurel are also used in veterinary medicine in crows and mares to facilitate removal of afterbirth[3].

Genetic variability of *L. nobilis* was previously studied[8]. The nuclear microsatellite DNA markers (nSSRs) were optimized and used to study the range wide genetic structure of *L. nobilis* in the Mediterranean Basin. The statistical analyses allowed us to identify two significantly different phylogenetic groups supported by strong bootstrap values, one including samples from France, Tunisia and Algeria, western Mediterranean and the other including two populations from Turkey, eastern Mediterranean[8].

The purpose of this study was to quantify the variation of laurel by some morphological and chemical (amount of essential oils) characters in relation to different regions in Tunisia. Moreover, we have studied antibacterial activity of essential oils extracted from leaves, with the aim of proposing strategies of exploitation and protection of this species.

EXPERIMENTAL

Leaves were sampled from ten *L. nobilis* L. trees in different stations of Tunisia. Codes of the samples are: Tn1. Sejnane; Tn 2. Ain Draham; Tn 3. Ain Elkhassa; Tn 4. Ain Snoussi 1; Tn 5. Ain Snoussi 2; Tn 6. Ain Essobeh; Tn 7. La Marsa; Tn 8. Rades; Tn 9. Hammamet; Tn 10. Sousse.

Morphological analysis: The ten sampled trees were used for Laurel morphological variability study. We have measured eight morphological descriptors. Morphological characters

were related to both vegetative and reproductive systems. Six branches of each tree were collected with reason of two ones from three different levels (basal parts, middle and apex). Moreover, we have studied the leaves dimensions. The leaves were also taken at three levels on the same branch (basal parts, middle and apex), two leaves of each branch level, so a total of 36 leave per tree. Lengths and widths were measured with a ruler on photocopied paper glued on fresh leaves. Leaf area was measured on a digital planimeter photocopy which has an accuracy of 0, 2 %. The values are the mean of three measurements.

Isolation of oils: We have collected about 2 kg of leaves from each tree at each site above mentioned. Leaves were separated from the lignified parts and air dried in the shadow for 15 days. Voucher specimens of each sample were deposited in the herbarium of the Faculty of Pharmacy in Monastir (Tunisia). 100 g of dried leaves boorishly crushed and mixed with 600 mL distilled water were subjected to hydrodistillation (HD) for 4 h using a modified Clevenger-type apparatus described[9].

GC/MS analysis: An Agilent Technologies Inc. gas chromatograph (Santa Clara, CA, USA) model 6890N was employed for analysis of the essential oils. It was equipped with a split-splitless injector, an autosampler Agilent model 7683 and an Agilent HP5 fused silica column; 5 % phenylmethylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 µm. GC conditions used were: programmed heating from 60 to 280°C at 3 °C/min, followed by 0.5 h under isothermal conditions. The injector was maintained at 250 °C. Helium was the carrier gas at 1.0 mL/min; the sample (1 µL) was injected in the split mode (1:20). The GC was fitted with a quadrupole mass spectrometer (MS, Agilent model 5973 detector). MS conditions were as follows: ionization energy 70 eV, electronic impact ion source temperature 200 °C, quadrupole temperature 100 °C, scan rate 1.6 scan/sec, mass range 50-500 µ. The software adopted to handle MS and chromatograms was a ChemStation NIST02 and LIBR (TP)[10,11]. Mass Spectra Libraries were used as references. Samples were run in chloroform with a dilution ratio of 1:100.

Identification of compounds: Compounds were identified by matching their MS and retention index with those reported in the literature[10,11]. Whenever possible, identification was confirmed by injection of an authentic sample of the compound. A quantitative analysis of each oil component (%) was carried out by peak area normalization measurement. The response factors were estimates using standard compounds having the same molecular weight as the compound families that constitute the essential oil (hydrocarbon and oxygenated monoterpenes, hydrocarbon and oxygenated sesquiterpenes).

Antibacterial Testing: The antibacterial activity of the different essential oils was evaluated by the paper-disc agar diffusion method[12] against the two Gram-negative model bacteria *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) and the two Gram-positive bacteria *Staphylococcus aure*us (ATCC 25923) and *Enterococcus faecalis* (ATCC 29212). Microorganisms were obtained from the culture collection of the Laboratory of Transmissible Disease and Biologically Active Substances, Faculty of Pharmacy of Monastir, Tunisia. Organisms were maintained on Muller-Hinton agar (MH) (BIORAD) medium. Inocula were prepared by diluting overnight (24 h at 37 °C) cultures in Muller Hinton Broth medium to approximately 10^6 CFU/mL. Absorbent discs (Whatman No. 3 discs, 6 mm diameter) were impregnated with 10 µL of oil and then placed on the surface of inoculated plates (90 mm). Positive control discs of gentamycine (10 µg/disc) were included in each assay. Diameters of growth inhibition zones were measured after incubation at 37 °C for 24 h.

Statistical analysis: We ran a principal component analysis (PCA) on a correlation matrix of a data set composed of 10 stations of Tunisia with 8 variables that concern the morphological characters: number of leaves by knot (NL/K), number of inflorescences by twig (NI/T), length of leaf (LL), length of twig (LT), width of leaf (WL), foliar surface (FS), number of flowers by twig (NF/T), number of leaves by twig NL/T). All statistical analyses are carried out using SPSS for windows v. 11.5(SPSS Inc., 2002).

RESULTS AND DISCUSSION

Morphology: Principal component analysis yields three principal components (PC1, PC2 and PC3), with Eigen values: 1.2, together they explain 89 % of the total morphological variation of Tunisian *Laurus nobilis* (Table-1). The first PC (49.19 %) has high positive loadings of number of inflorescences by twig, number of leaves by twig, length of leaf, width of leaf and foliar surface but negatively among number of flowers by twig. The second PC (29.02%) is defined positively by length of twig, number of inflorescences by twig, number of flowers by twig, the foliar surface and negatively by length of leaf. The loadings on the third PCA (10.91%) are dominated by length of twig, number of flowers by twig and width of leaves at the positive side.

It appears that two main groups of populations were clearly separated (Figs. 1 and 2). A first group included only female *L. nobilis* trees (2, 6, 8 and 10) and the other one included males and females. Aggregation of trees according to their geographical origin seems not obvious. Among female feet at Hammamet (10) and sejnane (1), the leaves are characterized by their reduced size. At Ain Essnoussi 1 (4), Ain

TABLE-1
LOADING PLOT OF THE STUDIED VARIABLES ON THE THREE PRINCIPAL COMPONENTS (PC1, PC2 AND PC3). NUMBER OF LEAVES BY KNOT (NL/K), NUMBER OF INFLORESCENCES BY TWIG (NI/T), LENGTH OF LEAF (LL), LENGTH OF TWIG (LT), WIDTH OF LEAF (WL), FOLIAR SURFACE (FS), NUMBER OF FLOWERS BY TWIG (NF/T), NUMBER OF LEAVES BY TWIG NL/T)

	(%)	Correlated parameters
Axe1	49.19	NI/T (+), NF/T (-), NL/T (+), LL (+), WL (+), FS (+)
Axe2	29.02	LT (+), NI/T (+), NF/T (+), LL (-), SF (-)
Axe3	10.91	LT (+), NF/T (+), WL (+)

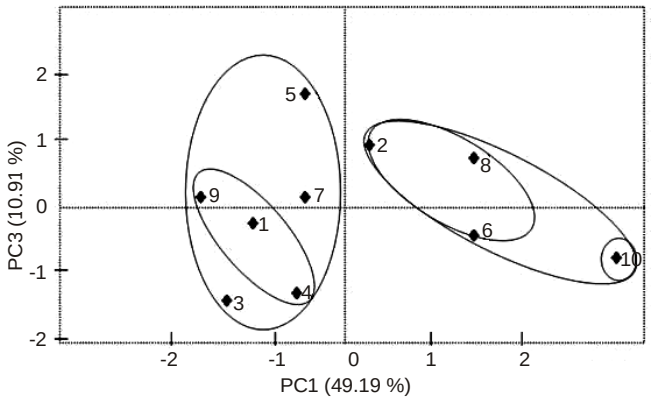

Fig. 1. Site score plot in the (PC1, PC2)-plane; 1:Tn1 (Sejnane); 2:Tn2 (Ain Draham); 3:Tn3 (Ain Elkhassa); 4:Tn4 (Ain Snoussi1); 5:Tn5 (Ain Snoussi2); 6 : Tn6 (Ain Essobeh);7:Tn7 (La Marsa);8:Tn8 (Rades);9:Tn9 (Hammamet);10:Tn10 (Sousse)

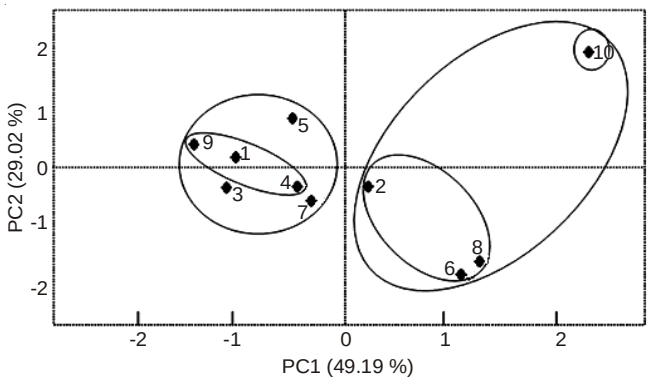

Fig. 2. Site score plot in the (PC1, PC3)-plane; 1:Tn1 (Sejnane); 2:Tn2 (Ain Draham); 3:Tn3 (Ain Elkhassa); 4:Tn4 (Ain Snoussi1); 5:Tn5 (Ain Snoussi2); 6 : Tn6 (Ain Essobeh);7:Tn7 (La Marsa);8:Tn8 (Rades);9:Tn9 (Hammamet);10:Tn10 (Sousse)

Essobeh (6) and Sousse (10), the trees present a very low number of inflorescences by knot and a much reduced numbers of flowers by twig. On the other hand, female trees present at Sejnane (1), Aïn Draham (2), Radès (8) and Hammamet (9) are characterized by very important inflorescences comparable to those of the male trees (Ain Elkhassa (3), Ain Essnoussi 2 (5) and Marsa (7). Although trees originated from the same bioclimatic zone at Ain Essnoussi 2 (5) opposes to Ain Essnoussi 1 (4) and Ai Elkhassa (3) sites by the reduced length of twig and the low number of leaves by twig. Morphological and chemical characters of plants are determined genetically, but many quantitative features may vary under different environmental conditions of habitats and so determine the interpopulation variation of species[13].

Essential oils: To evidence a probable chemical variability, the study was carried out on ten individuals from leaves sampled from *L. nobilis* L. trees in different stations of Tunisia. The essential oils were isolated by hydro-distillation and yields ranging from 0.9 to 2.2 % (w/w) were obtained. In total, 54 components, representing 97.4 to 99.3 % of the composition of the oil samples, were identified by comparison of their retention indices and mass spectral patterns with those of reference compounds compiled either in spectral data libraries or in the literature (Table-2).

Although all the oil samples could be classified as monoterpene-rich oils, their composition varied slightly. Indeed, a few oxygenated monoterpenes dominated the oil samples composition, 1,8-cineole (up to 38.9%), methyl eugenol (up to 18.8%), α-terpinyl acetate (up to 15.4%), sabinene (up to 8.0%) and α/β-pinene (up to 5.8 and 4.2 %, respectively). Beside monoterpenes, two sesquiterpenes (spathulenol and α-cadinol) were present in moderate amounts in almost all the samples. A little variability was observed with respect to the fifty other minor and trace constituents of leaves *L. nobilis* L. oils. The results from this study show that oils obtained from leaves have nearly similar compositions that Mediterranean and European *L. nobilis*[14-18].

The study[19] showed that main components of the essential oils were 1,8-cineole, *trans*-sabinene hydrate, α-terpinyl acetate, methyl eugenol, sabinene, eugenol and α-pinene extracted from aerial part at different phonological stage of Iranian *Laurus nobilis* L. development.

Antimicrobial activity: The antimicrobial activities of *L. nobilis* L. essential oils originating from Tunisia were evaluated by a paper disc diffusion method against tested bacteria. As shown in Table-3, all essential oils were active against the *S. aure*us and *E. faecalis* with the largest inhibition zones ranged between 10 to 20 and 10 to 14 mm, respectively. Except the essential oil of Rades region, the rest exhibited an interesting activity against *E. coli* (10 to 20 mm). On the other hand we notice the resistance of the bacterium *Pseudomonas* (Gram-) with the majority of the samples of essential oil, only the oils from Hammamet and Ain Essoussi 2 regions showed the lowest inhibition zones of 10 mm against *P. aeruginosa*. In other studies of Gram-negative bacteria, *Pseudomonas* and in particular *P. aeruginosa*, appear to be least sensitive to the action of essential oil [20-22]

From our results, related to the inhibition of growth, significant differences were detected among these cited oil types, since all of them showed, relatively, a similar composition. As cited in the literature, phytochemicals including 1,8-cineole offer a source of new compounds with interesting antibacterial activity. In this preliminary study, the oil composition alone, cannot explain the results of the diffusion disc tests that suggest methyl eugenol, pinene, linalool and sabinene were primarily responsible for the activity with 1,8-cineole less active. This variability could be due to the diffe-rence in the levels of their major and minor components and to a synergetic effect between all components. This finding was in agreement with previous reports[23-25].

Conclusion

Among individual plants of *Laurus nobilis* L. originating from various Tunisian localities, two main groups of populations were detected. The leaves of the group including only the female feet are characterized by their reduced size, low number of inflorescences by knot, reduced numbers of flowers by twig and by very important inflorescences when compared to those of the male trees. In all analyzed samples, the leaves essential oils showed, relatively, a similar composition rich in oxygenated terpenes revealing a potent therapeutic uses. However, further research is needed to investigate the bio-activity and toxicity of these essential oils using *in vivo* trials.

TABLE-2
CHEMICAL COMPOSITION (%) OF THE ESSENTIAL OILS OF TEN *Laurus nobilis* L. TREES IN DIFFERENT STATIONS OF TUNISIA

Compound	Tn1	Tn2	Tn3	Tn4	Tn5	Tn6	Tn7	Tn8	Tn9	Tn10
Tricyclene	0.6	0.5	0.5	0.5	0.6	0.5	0.6	0.5	0.5	0.5
α-Pinene	4.5	3.3	4.2	5.8	4.6	3.7	3.5	3.9	4.2	4.3
Camphene	0.4	tr.	1.3	1.6	0.7	0.3	0.3	0.7	0.5	0.7
Sabinene	8.0	5.1	4.2	6.2	5.7	5.5	4.2	5.6	5.6	5.9
β-Pinene	3.3	2.6	3.2	4.2	3.3	2.7	2.5	2.7	3.0	3.2
Myrcene	0.7	0.4	0.2	0.5	0.5	0.5	0.3	0.5	0.3	0.4
α-Phellandrene	tr.	tr.	tr.	0.4	tr.	tr.	tr.	tr.	tr.	tr.
Δ2-Carene	0.6	0.7	1.3	tr.	0.3	0.5	1.8	tr.	tr.	0.2
Δ3-Carene	tr.	0.5	0.5	0.6	0.6	tr.	0.4	0.4	0.6	0.6
o-Cymene	tr.	tr.	0.3	tr.	tr.	0.2	0.6	tr.	tr.	tr.
p-Cymene	tr.	0.5	0.7	0.3	tr.	0.5	1.5	0.2	tr.	0.2
1,8-Cineole	34.5	30.3	30.5	34.5	35.5	38.9	37.8	25.9	30.3	30.8
z-β-ocimene	0.2	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
γ-Terpinene	1.0	1.1	0.9	1.1	1.0	0.9	0.8	0.7	1.1	1.0
cis-Sabinene hydrate	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.4	0.3
Terpinolene	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.4	0.3
Linalool	1.7	2.5	3.4	4.1	1.5	5.6	2.0	9.7	7.9	6.1
trans-Pinocaveol	tr.	tr.	tr.	0.1	tr.	tr.	0.3	tr.	tr.	tr.
Borneol	tr.	tr.	1.4	2.5	1.2	tr.	tr.	tr.	0.4	0.2
p-Mentha-1,5-dien-8-ol	0.7	0.3	tr.	tr.	tr.	0.5	0.5	0.6	0.5	0.4
Terpin-4-ol	2.4	3.4	3.3	3.0	3.1	3.6	4.6	2.2	2.8	2.8
α-Terpineol	2.5	1.9	2.1	3.6	3.6	2.1	2.1	3.0	5.2	2.1
cis-Sabinenehydrate acetate	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
Nerol	0.3	0.3	0.3	0.3	tr.	0.2	0.2	0.3	tr.	0.3
Linalool acetate	tr.	tr.	tr.	tr.	tr.	0.3	tr.	0.3	tr.	0.2
Bornyl acetate	0.3	tr.	1.2	0.1	tr.	0.5	0.3	0.9	tr.	0.8
Iso-3-thujyl acetate	0.6	0.7	0.6	0.5	0.7	0.8	0.7	0.5	0.5	0.7
α-Terpinyl acetate	15.4	14.3	12.9	13.4	12.8	14.5	13.6	10.9	11.4	14.5
Eugenol	2.9	0.5	3.5	1.2	1.5	2.3	1.9	2.4	2.0	2.2
α-Yalangene	tr.	tr.	tr.	tr.	tr.	tr.	0.2	tr.	tr.	tr.
β-Cubebene	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
β-Longipinene	0.8	0.8	0.5	0.1	0.6	0.3	0.3	0.7	0.6	0.4
Methyl eugenol	10.0	18.8	14.3	11.3	16.3	6.3	11.6	15.2	12.8	15.6
α-Gurjunene	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
E-Caryophyllene	1.1	1.1	0.7	0.1	0.7	0.6	0.7	0.6	1.0	0.4
α-Guaiene	tr.	tr.	tr.	tr.	tr.	tr.	tr.	0.2	tr.	tr.
cis-Muurola-3,5-diene	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
α-Himachalene	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
Germacrene D	0.2	0.6	0.2	tr.	tr.	tr.	0.2	tr.	0.5	0.4
cis-β-Guaiene	tr.	0.3	tr.	tr.	tr.	tr.	0.2	0.3	0.3	tr.
Bicyclogermacrene	0.6	0.5	0.5	tr.	0.4	0.3	tr.	1.2	tr.	0.4
Viridiflorene	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
α-Bulnesene	tr.	0.3	tr.	tr.	tr.	tr.	tr.	0.4	tr.	tr.
trans-Cadinene	tr.	0.5	tr.	0.1	tr.	tr.	tr.	tr.	tr.	tr.
δ-cadinene	0.4	0.7	tr.	0.2	0.4	0.4	0.4	0.4	0.6	0.3
Elemicin	0.7	1.4	0.5	0.4	0.6	0.8	0.7	0.8	1.0	0.5
Spathulenol	1.5	1.0	1.3	0.5	0.7	1.7	0.7	2.2	0.9	1.0
caryophylene oxide	1.0	0.7	0.7	0.2	0.7	1.2	1.0	0.7	0.8	0.6
Globulol	tr.	0.4	0.2	tr.	tr.	0.2	tr.	0.3	tr.	tr.
N.I.	tr.	0.3	tr.	tr.	tr.	tr.	tr.	0.2	tr.	tr.
N.I.	tr.	0.2	0.3	0.1	tr.	tr.	tr.	tr.	tr.	0.3
1-Epicubinol	tr.	0.5	0.3	tr.	tr.	0.4	0.7	0.3	0.2	tr.
β-Eudismol	tr.	tr.	tr.	tr.	0.3	tr.	0.4	0.4	1.1	0.2
α-Cadinol	0.7	1.1	0.7	0.1	0.6	0.6	0.4	0.6	0.6	0.4
z-Nerolidol acetate	tr.	tr.	0.2	tr.	tr.	tr.	tr.	tr.	tr.	tr.
5-Izocedranol	0.5	0.7	0.3	0.1	tr.	0.4	tr.	0.5	tr.	0.1
Total identified	98.9	99.3	97.8	98.3	99.1	98.4	98.6	97.4	98.0	99.3

tr: trace, i.e., percentage lower than 0.1%; N.I.: not identified compound.

TABLE-3
ANTIMICROBIAL ACTIVITY OF THE INVESTIGATED ESSENTIAL OILS AND THE STANDARD ANTIBIOTIC (GENTAMICIN) AGAINST FOUR MODEL BACTERIA (INHIBITION ZONE DIAMETERS: mm)

	Microorganisms			
	S. aureus ATCC25923	*E. coli* ATCC25922	*P. aeruginosa* ATCC227853	*E. faecalis.* TCC292112
Tn1	11	10	Without effect	11
Tn2	13	13	Without effect	10
Tn3	13	13	Without effect	12
Tn4	15	14	Without effect	11
Tn5	20	20	10	10
Tn6	14	12	Without effect	12
Tn7	12	10	Without effect	14
Tn8	10	Without effect	Without effect	10
Tn9	10	10	10	10
Tn10	10	11	Without effect	10
Gentamycine (10 µg/mL)	25	20	21	20

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